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Intrabody Construction and Expression. II. A Synthetic Catalytic Fv Fragment

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In general, proteins with structural disulfides cannot be expressed in the reducing environment of the cellular cytoplasm. To overcome this folding problem, we have previously engineered stabilizing mutations, predicted from a consensus sequence analysis, into isolated immunoglobulin V_L domains. Here we show that such domains can be used as a framework in the construction of a functional heterodimeric Fv fragment, which was expressed solubly, with high yield in the cytoplasm of *Escherichia coli*. This designed catalytic intrabody, obtained from grafting the combining site of the esterolytic antibody 17E8, is active in the oxidized and the reduced state. Its construction required no special features on the part of the immunoglobulin, no single-chain linker and introduced no non-natural sequence motifs. The potential to design intrabodies with the recognition sequences of arbitrary immunoglobulins opens novel opportunities for gene therapy, cell biology, metabolic engineering and antibody biotechnology.

Keywords: intrabodies; immunoglobulin engineering; protein engineering; protein stability; catalytic antibodies

Introduction

The recognition epitopes of antibodies are located in hypervariable segments of the so-called Fv fragment, a heterodimer comprising a variable domain of the light chain (V_L) and the heavy chain (V_H). Recombinant Fv fragments are in use throughout the life sciences as specific, high-affinity ligands for analysis (Carter & Merchant, 1997), therapy (Hudson, 1998; Marasco, 1997) or even catalysis (Schultz & Lerner, 1995; Smithrud & Benkovic, 1997). In contrast to their widespread use, the most important compartment of the cell, i.e. the cellular cytoplasm, in general has been *terra incognita* for the application of immunoglobulin domains. The reason is a strictly conserved structural disulfide bond within each domain that cannot be formed in the reducing environment of the cytoplasm (Biocca *et al.*, 1995). This bond alone typically contributes more than the net free energy of folding to each domain's stability. As a general rule, attempts to express functional antibody domains in a reducing environment fail (Glockshuber *et al.*, 1992) and chemical reduction denatures the domain *in vitro*. Some exceptions have been reported, notably intracellular single chain Fvs (scFvs) against HIV proteins (Maciejewski *et al.*, 1995; Wu *et al.*, 1996; Rondon & Marasco, 1997), but low stability, low expression rates and unpredictable behavior currrently limit these perspectives (Marasco, 1997; Gargano & Cattaneo, 1997). Indeed, most of the material expressed cytoplasmically in mammalian cells will form insoluble aggregates (Cattaneo & Biocca, 1999). The fact that the use of such proteins in clinical trials is currently being actively pursued (Marasco *et al.*, 1998), emphasizes the need to better understand the folding and stability of immunoglobulin domains under reducing conditions.

Recently, two groups have applied methods of evolutionary engineering to the generation of intrabodies and obtained functional, reduced scFvs by random mutagenesis and screening. Starting from an antibody that naturally lacks Cys92 in V_H, an scFv-fragment was evolved by extensive rounds of DNA-shuffling and phage display selection (Proba *et al.*, 1998). High levels of soluble cytoplasmic expression were also reported for an scFv-

Abbreviations used: CDR, complementarity-determining region; Fv, heterodimer containing V_L and V_H; scFv, single chain Fv fragment; GdmCl, guanidinium chloride; ΔG_F, free energy of folding; NFMP, N-formyl methionine-phenylester; PBS, phosphate-buffered saline; V_L and V_H, variable domains of immunoglobulin light and heavy chains, respectively.

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fragment, directed against a modified β-galactosidase (Martineau *et al.*, 1998). Here, domains were pre-selected by *in vitro* screening and expressed in host cells subjected to a protocol for *in vivo* selection of functional antibodies. However, these scFvs were either derived from one of the rare immunoglobulins which have somehow compensated for the loss of their conserved disulfide bond† *in vivo*, or assayed and evolved under reducing conditions in a genetic screen for that specific antibody's function. The generalization of these methods to arbitrary immunoglobulin domains does not appear to be straightforward. Indeed, a recent loop-grafting experiment onto a naturally stable scFv fragment for the purpose of intracellular expression failed to yield soluble protein in the cellular cytoplasm (Wörn & Plückthun, 1998).

Thus the problem arises to define a general approach to compensate for the absence of the structural disulfide bridge in the cytoplasm. To be practical, the required level of stability of the immunoglobulin should not depend on the availability of the ligand to form stable complexes. Furthermore, we need to investigate whether an increase of folding stability alone is sufficient to express a functional heterodimeric Fv fragments in the cytoplasm, or whether the cellular environment further limits accession of the native state on the altered folding pathway of reduced domains, which would necessitate engineering of these less-well understood aspects of protein folding. Finally, mutations introduced in the engineering process, as well as the reduced disulfide bond itself, should not interfere with the domain function.

Here we show that a combination of stabilizing mutations, derived from a consensus sequence analysis, provides a general and plannable route to the cytoplasmic expression of a functional intrabody Fv-fragment in high yields.

Results

We have previously addressed the problem of increasing folding stability in a rational way (Steipe *et al.*, 1994). Applying a strategy based on engineering consensus sequences, the stability of isolated V_L domains was improved to the point where they can be solubly expressed in the cellular cytoplasm (Ohage & Steipe, 1999). For the design of a stabilized catalytic antibody fragment for cytoplasmic expression, we chose a heterodimeric Fv fragment rather than a single-chain Fv, in order to characterize the behavior of individual V_L as well as V_H domains and to be able to distinguish association effects from effects on domain folding. The functional epitopes were derived from 17E8 (Zhou *et al.*, 1994), a well-characterized esterolytic antibody. Since the hapten is not normally present in the cell, complex formation does not contribute to domain stabilization.

To construct the catalytic intrabody Ica-Fv we first introduced the relevant CDR sequences of the light chain of 17E8 into the framework of our hyperstable V_L domain V_L-601 (Ohage & Steipe, 1999), by exchanging those residues which are a part of the antigen binding site (Figure 1). Furthermore, serine 7 was deleted in order to force *cis*-proline 8 into a *trans*-conformation. This introduces a λ-chain consensus motif and simplifies the folding pathway. The resulting V_L chain was called IcaL-14. Equilibrium unfolding transition curves for IcaL-14 were concentration dependent, indicating domain dimerization. We attribute this to the unusual CDR3 region of the V_L domain and the absence of an aspartic acid residue which presumbly prevents dimerization in the parent V_L domains of McPC603 (Steipe *et al.*, 1992). Assuming a two-state model of unfolded monomer and folded dimer (Gittelman & Matthews, 1990), the thermodynamic stability ΔG_F for IcaL-14 can be determined as −78.1(±1.2) kJ/mol (Figure 2). After chemical reduction of the disulfide bridge in IcaL-14, ΔG_F was −59.1(±2.0) kJ/mol. Thus, the energy loss caused by reduction is 19.0 kJ/mol, less than twice that of the monomeric V_L-601, for which the loss in folding energy accompaying reduction was 15.5 kJ/mol (Ohage & Steipe, 1999). Even though folding and association energies cannot be separated in this analysis, dimer transition curves are similar to those of the monomer (Figure 2). Compared to the monomeric domain, it appears that IcaL-14 is more susceptible to denaturation in the oxidized state but less so in the reduced state.

To construct the corresponding V_H domain IcaH-01, we used the V_H domain of the anti-para-nitrophenyl antibody B1-8 (Bothwell *et al.*, 1981) as a scaffold. This domain already contains most residues of the murine V_H consensus sequence but it cannot be expressed in soluble form in the cytoplasm (B.S., unpublished results). The same was observed for isolated IcaH-01 domains. In contrast, the coexpression of IcaH-01 with IcaL-14, and secretion into the periplasm of *E. coli*, was successful. This Fv fragment, Ica-Fv20, demonstrated completely reversible folding behavior (data not shown) with two well-discernible transitions for V_H and V_L (Wirtz & Steipe, 1999).

The hydrolysis rates of the secreted, oxidized Fv fragment for *N*-formyl methionine-phenylester were determined in PBS (pH 7.4). Activity of the disulfide-intact Fv fragment followed Michaelis-Menten kinetics with a K_M of 820 μM and a k_{cat} of 1.9 min^{-1}, resulting in a k_{cat}/K_M of 2.3×10^3 min^{-1} M^{-1} (Figure 3). This is about one-third of the activity reported for 17E8 at pH 7.2

† Indeed, the antibody framework A48 investigated by Proba and co-workers has undergone a major conformational change in one of the framework β-strands to compensate for the mutation that disrupts the disulfide bridge. The unpaired cysteine residue is turned outward, into the solvent (Proba *et al.*, 1997). A similar observation has been reported for scFv13, the β-galactosidase binding intrabody, in which the heavy chain disulfide bridge apparently does not form under oxidising conditions (Martineau *et al.*, 1998).

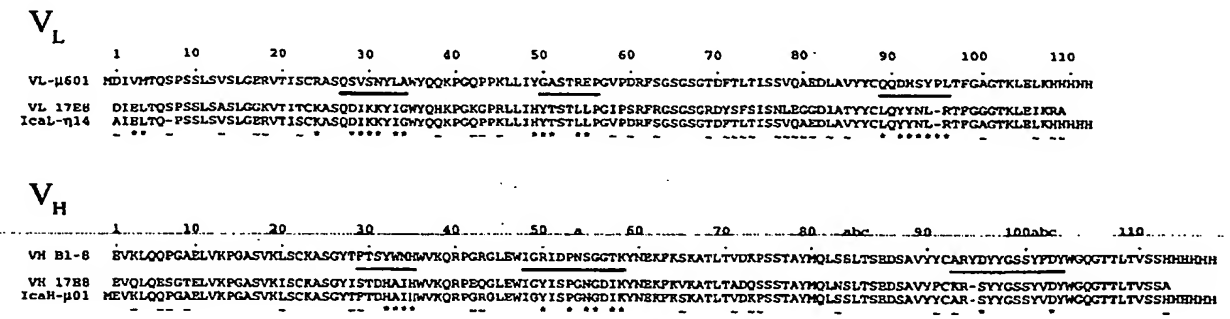

Figure 1. Sequences for the variable domains used in this study: V_L-μ601 and the catalytic intrabody Ica-Fv, and the variable domains of the antibody 17E8. CDRs of 17E8 are underlined, changes introduced into the respective framework sequences for grafting the catalytic epitopes are indicated with an asterisk (*), the remaining sequence differences between the catalytic 17E8 and Ica-Fv are emphasized with a (~). IcaL-η14 deviates in 29 positions, IcaH-μ01 deviates in 17 positions from the 17E8 sequence.

(Guo *et al.*, 1994), normalized to one active site†, and corresponds to a 4300-fold acceleration above background. Considering the large number of sequence differences between Ica-Fv and 17E8 (Figure 1), the observed difference in activity is remarkably small and emphasises the non-disruptive nature of consensus sequence mutations. Neither IcaL-14 nor IcaH-01 alone accelerate the hydrolysis rate above background (data not shown).

For cytoplasmic expression of Ica-Fv20, genes for IcaL-14 and IcaH-01 were amplified by PCR without a leader sequence (IcaL-η14 and IcaH-μ01‡) and cloned into the cytoplasmic expression vector pIcaFv20c (Figure 4). Purified protein could be successfully recovered from the soluble fraction of the cytoplasm in quantities of 2 mg/l of a shake flask culture (Figure 5). The failure of attempts to express isolated IcaH-μ01 in the cytoplasm demonstrates a stabilizing interaction of V_L with V_H in the cytoplasm in the absence of a peptide linker.

Finally, to determine whether the introduction of stabilizing mutations and the reduced cysteine residues in the core influence the Fv fragment's structure and function, we measured the catalytic activity of cytoplasmically expressed IcaFv20 under reducing conditions (0.5 mM β-mercaptoethanol). We determined a K_M of 126 μM and a k_{cat} of 1.6 min^{-1}, which corresponds to a k_{cat}/K_M of 12.7×10^3 min^{-1} M^{-1} (Figure 3). This protein differs in one N-terminal residue in each chain from the periplasmatically expressed sequence and constitutes the functional cytoplasmic form. While the catalytic activity agrees well with the value obtained for the oxidized Fv-fragment, K_M is even slightly smaller in the reduced state.

† K_M for the antibody 17E8 at pH 7.4 was not given in the reference (Guo *et al.*, 1994), k_{cat} was 13.4 min^{-1}, 6.7 min^{-1} per active site.

‡ The prefixes η and μ refer to different N termini; see Ohage & Steipe (1999).

Discussion

Rationally predicted, stabilizing mutations can be combined to construct hyperstable frameworks for heterodimeric immunoglobulin domains, on which the recognition epitopes of other antibodies can be grafted without significant change in function. These domains possess sufficient folding energy to compensate for the loss of the structural disulfide bridge and can be expressed solubly, with high yield and in functional form in the cellular cytoplasm. The presence of the antigen or the introduction of a peptide linker to promote domain association is not required. From our results it appears that an approximation of the respective consensus sequence for the domains, wherever allowed by the requirements for antigen binding, is sufficient to generate functional intrabodies. Since no residues that are highly untypical for immunoglobulin domains are required, problems of com-

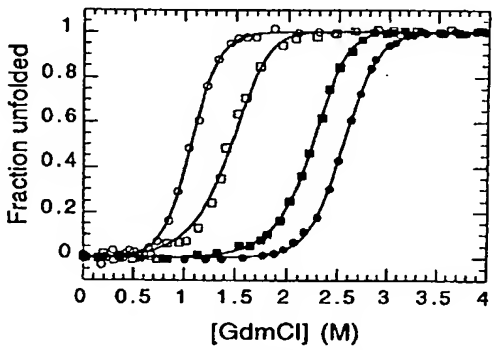

Figure 2. Normalized transition curves for the stabilized monomeric V_L domain V_L-601 (circles) and the homodimeric IcaL-14 (squares) at 20°C and pH 7.4. Filled symbols refer to oxidized domains (intact disulfide bridge), open symbols to reduced domains (free thiols). Protein concentrations were 2 μM referring to the monomer. Both domains fold fully reversibly under oxidizing and reducing conditions.

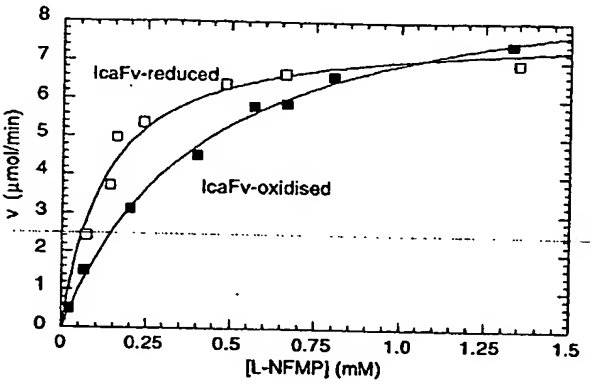

Figure 3. The v/[S] plot for the hydrolysis of L-NFMP by oxidized and reduced Ica-Fv at 20°C and pH 7.4. The concentration of Ica-Fv was 5µM. Curve fits and kinetic constants were calculated for simple Michaelis-Menten kinetics. For the oxidized fragment, K_M was 820 µM, k_{cat} was 1.9 min^{-1}, and k_{cat}/K_M was 2.3×10^3 min^{-1} M^{-1}. For the reduced fragment, K_M was 126 µM, k_{cat} was 1.6 min^{-1}, and k_{cat}/K_M was 12.7×10^3 min^{-1} M^{-1}.

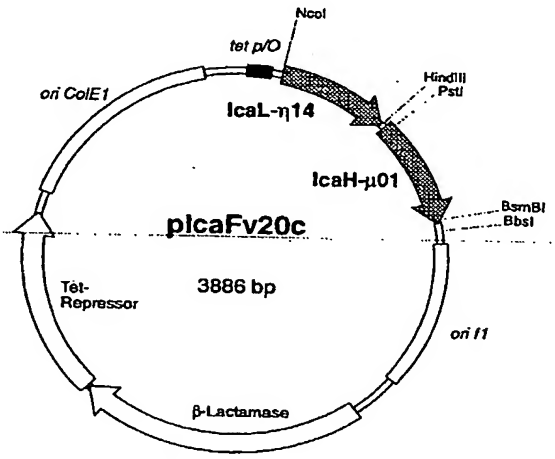

Figure 4. Plasmid map for the cytoplasmic expression vector pIcaFv20c. The variable domains (gray) are inserted into a plasmid backbone derived from pASK75 Skerra, 1994) and expressed from a dicistronic operon under control of the *tet*A-promotor/operator. Singular restriction sites useful for cloning are shown.

bining the stabilized framework with arbitrary recognition epitopes are minimized. Indeed, it may be advantageous for therapeutic purposes, that no novel sequence motifs are introduced; it is one of the corollaries of the canonical sequence approximation, that while each individual sequence has only a low probability of occurring in the ensemble, discrete consensus epitopes occur in the ensemble with a high probability. Thus consensus engineering may even lead to synthetic immunoglobulin domains with a decreased allergenic potential. The fact that the individual mutations are only weakly context dependent makes consensus sequence engineering a general approach; this is evidenced by the successful application to immunoglobulin domains with no special features or provenance that would make them predestined for cytoplasmic expression.

Functional immunoglobulin fragments that fold under reducing conditions, or which no longer require cysteine residues for folding, possess a number of significant advantages for biotechnological processes. The construction and expression of fusion proteins is simplified, fusions with cytoplasmic proteins can be constructed and expression levels can be two to three orders of magnitude larger for cytoplasmic expression than for periplasmatic secretion. Most importantly though, we expect our approach to make intrabody technology widely available for analytic, biotechnological or therapeutical purposes, e.g. for the immunolocalization of targets *in vivo*, for modulating the concentration or activity of cellular targets by binding or sequestration into specific cellular compartments, for modulating the cellular metabolism with catalytic antibodies, or to harness the power of intracellular genetic screens for the enginering of new antibody specificities.

Methods

Consensus sequences and vector construction

Consensus sequences were derived from the fifth distribution of the Kabat Immunoglobulin Sequence Database (Kabat *et al.*, 1992; B. Steipe, 1998, *The Canonical Sequence Approximation*, http://www.lmb.uni-muerche.de/groups/bs/canonical.html). Periplasmic expression vectors were constructed *via* site-directed mutagenesis using standard technologies as described (Ohage *et al.*, 1997). For cytoplasmic expression, V_L and V_H genes were amplified by PCR to remove the leader peptide and cloned into an expression vector derived from pASK75 (Skerra, 1994; Ohage & Steipe, 1999).

Protein expression and purification

For oxidative folding, proteins were expressed in *E. coli* JM83, secreted into the periplasm, harvested from the periplasmic cell fraction and purified to homogeneity by immobilized metal ion affinity chromatography as described (Ohage *et al.*, 1997). For cytoplasmic expression, *E. coli* JM109 cells harboring the respective plasmids were grown to an A_{600} of 0.9, induced by the addition of 430 nM anhydrotetracycline, further incubated overnight at 20°C and harvested. All the following steps were performed in the presence of 1 mM β-mercaptoethanol to ensure reducing conditions. The cells were disrupted in a French press, centrifuged at 48,000 rcf for 20 minutes and the supernatant was subjected to immobilized metal ion affinity chromatography on a Ni^{2+}-nitrilo-triacetic acid matrix (Qiagen GmbH, Hilden, Germany). Immunoglobulin domains were eluted with an imidazole gradi-

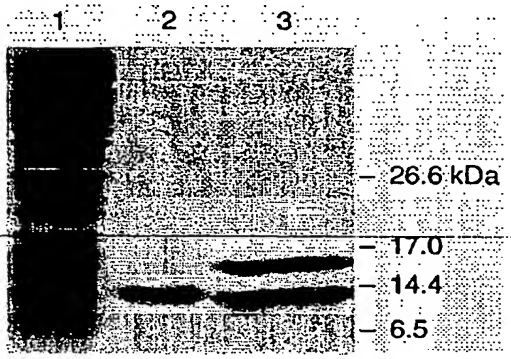

Figure 5. Intracellular expression and purification of Ica-Fv under reducing conditions. The SDS-PAGE shows the soluble cellular supernatant, applied to an IMAC column (lane 1), cytoplasmically expressed V_L-μ601 protein as standard (lane 2) and pooled fractions of cytoplasmically expressed Ica-Fv, eluted with an imidazole gradient (lane 3).

ent. SDS-PAGE and subsequent Coomassie-staining showed that V_L was more abundant than V_H but could be separated as it eluted before the heterodimeric Fv fragment. To obtain the Fv-fragment, fractions with stoichiometric amounts of V_L and V_H were pooled and concentrated. One further purification step with an anion exchange resin was necessary to remove a contamination commonly copurified from the cytoplasm under these conditions (Wülfing *et al.*, 1994). The protein yield was determined spectrophotometrically at 280 nm. Coefficients of absorption were estimated from the amino acid composition to be 14,650 $M^{-1}cm^{-1}$ for IcaL-η14 and 29,870 $M^{-1}cm^{-1}$ for IcaH-μ01(Gill & von Hippel, 1989).

Protein purity

The expressed domains were analyzed for purity by SDS-PAGE gels and no contaminant protein bands were seen after silver staining (Figure 5). To demonstrate homogeneity of the preparation, an aliquot of Ica-Fv from cytoplasmic expression (stored in PBS, 0.5 mM β-mercaptoethanol) was brought to a concentration of 20 μM, dialyzed against water and diluted with three volumes of 50% methanol, 1% acetic acid. Mass spectra were taken on a Finnigan MAT TSQ 7000 spectrometer and the calculated molecular masses were 12,222.8 Da (expected, 12,223.7, N-terminal Met processed) for IcaL-η14 and 14,101.7 Da (expected, 14,102.2, N-terminal Met present) for IcaH-μ01.

Determination of folding stability

Unfolding was achieved by diluting protein stocks into phosphate-buffered saline solution (115 mM NaCl, 16 mM Na_2HPO_4, 4 mM KH_2PO_4, pH 7.4) containing the indicated concentrations of guanidinium chloride (GdmCl). Protein concentration was 2 μM. The temperature of incubation and measurement was 20°C. For *in vitro* reduction of secreted, oxidized protein, the material was unfolded in PBS containing 4 M GdmCl and 50 mM DTT and diluted into PBS-GdmCl containing 3 mM DTT. Unfolding was monitored by a fluorescence increase at 350 nm (oxidized domains) or a decrease at 320 nm (reduced domains) after excitation at 280 nm. Experimental values were fitted to equations derived from a two-state linear free energy model involving unfolded and folded monomer ($U \rightleftharpoons N$, $K = [N]/[U]$, Fraction unfolded $= F_U = 1/1 + K$) or unfolded monomer and folded dimer ($2U \rightleftharpoons N_2$, $K = [N_2]/[U]^2$, $F_u = \sqrt{8P_{tot}K + 1} - 1/4P_{tot}K$), where K is the equilibrium constant and P_{tot} the protein concentration in terms of monomer.

Catalytic activity

Hydrolysis velocities were determined at 20°C by the increase of the A_{270} after diluting stock solutions of racemic *N*-formyl methionine-phenylester (NFMP) with oxidized Fv fragment in PBS (pH 7.4), or reduced Fv fragment in PBS containing 0.5 mM β-mercaptoethanol, as compared to the background reaction (Figure 3). The concentration of the Fv fragment was 5 μM, the concentration of the ester was determined photometrically after total hydrolysis in NaOH and divided by two, since the catalyzed reaction is specific for the L-enantiomer (Guo *et al.*, 1994).

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